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RESEARCH ARTICLE

STUDY ON KLEBSIELLA PNEUMONIAE CARBAPENEMASE AND METALLO BETA LACTAMASE ENZYMES IN CLINICAL ISOLATES OF GRAM NEGATIVE BACILLI

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ABSTRACT

Background: Resistance to broad spectrum β-lactams mediated by Extended spectrum β-lactamases and AmpC β lactamases is an increasing problem worldwide. The advent of carbapenems heralded a new treatment option for serious bacterial infection. But carbapenemases have emerged and spread, leading to carbapenem resistance .Hence, it is necessary to know their incidence in the clinical isolates of the hospital, so as to formulate a policy of empirical therapy in high risk patients.
Materials and methods: A total of 250 isolates of Gram negative bacilli isolated from various clinical samples received in the Department of Microbiology, BMC & RI were included in the study. Antibiotic susceptibility was done according to Kirby-Bauer disk diffusion method on Mueller Hinton agar and results were interpreted according to CLSI guidelines. Carbapenemases were screened and confirmed by CLSI recommended Modified Hodge test(MHT) and Phenyl boronic acid/Ethylene diamine tetra acetic acid (BA/EDTA) disk potentiation test.
Results: The most common organism isolated was Escherichia coli (44%) followed by Klebsiella pneumoniae (29.6%). 3.2%, 12.8% and 3.6% were pure KPC, pure MBL and co-producer of KPC and MBL respectively. The BA/ EDTA disk potentiation test is better than MHT for detection of carbapenemase and MBL.
Interpretation and Conclusion: The BA/ EDTA disk potentiation test is better than MHT for detection of carbapenemases and MBL and is a satisfactory and inexpensive method for characterizing the type of carbapenemase when PCR is not readily available. Thus the combined disc potentiation test could be used as a convenient screening method for carbapenemases.

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INTRODUCTION

Beta-lactamases are the enzymes produced by microorganisms which can open up/hydrolyze the β-lactam ring of β-lactam antibiotics like Penicillins and Cephalosporins. (Bush Karen, 1988) The selection pressure due to the use and overuse of newer antibiotics, especially Oxyimino-cephalosporins in the treatment of patients has resulted in the production of new variants of β-lactamases. These β-lactamases have been found worldwide in different genera of *Enterobacteriaceae* and other Gram-negative organisms. (Bradford, 2001) Carbapenems are distinctive substrates for serine carbapenemases and metallo β lactamases (MBL). Klebsiella pneumoniae carbapenemase (KPC) is a serine carbapenemase associated with major out breaks of multidrug resistant Gram negative infections worldwide.

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KPC confers resistance to all β lactam agents including penicillins, cephalosporins, monobactam and carbapenems and are inhibited better by tazobactam than clavulanic acid. (Bush Karen and Jacoby, 2010) Boronic acid compounds were reported as reversible inhibitors of Class C AmpC enzymes, class A (KPC) β lactamases, and some of the CTX- M-Type ESBLs. This inhibition is a structure based mechanism where boronic acid forms covalent bond with the active serine site of the enzyme. Recently they have also been evaluated for the differentiation of KPC producing *Enterobacteriaceae*. (Pournaras *et al*., 2010) Metallo β lactamases (MBL) are zinc containing enzymes characterized by their ability to hydrolyze carbapenems, penicillins and cephalosporins but not monobactams. They are inhibited by metal ion chelators like EDTA, dipicolonic acid, or 1, 10-O-phe-nanthroline and not inhibited by β lactamase inhibitors. (Bush Karen and Jacoby, 2010) Resistance to broad spectrum β-lactams mediated by ESBLs and AmpC β lactamases is an increasing problem worldwide. (Sinha *et al*., 2008)

The advent of carbapenems heralded a new treatment option for serious bacterial infection. (Walsh *et al*., 2005) But carbapenemases have emerged and spread, leading to carbapenem resistance. The only treatment option that remains is the potentially toxic Polymyxin B and Colistin. (Behera *et al*., 2008) Although there are new agents within existing classes of antimicrobials, currently there are no new classes of antimicrobials in the later phases of development with activity against MDR-Gram-negative bacteria. (Arnold *et al*., 2011) The polymyxins and tigecycline are highly active but have narrow therapeutic indices and pharmacokinetic profiles. Because no alternative antibiotics are currently available, it is likely that clinicians will turn to unconventional combinations to treat KPC infections. However, panresistant bacteria have been reported that are resistant to tigecycline, polymixin, and aminoglycosides. (Arnold *et al*., 2011) Monotherapy is associated with higher treatment failure rates compared to combination therapy when managing infections due to KPC-producing bacteria, particularly when treating respiratory infections. Polymyxins and carbapenems when used alone were associated with higher treatment failure than when used in combination. (Lee and Burgess, 2012) Carbapenems and polymyxins should probably not be used as monotherapy for infections caused by KPC bacteria. Combination therapy may improve outcomes and be helpful in preventing bacterial resistance. (Arnold *et al*., 2011)

Therefore this study is undertaken to detect carbapenemases in Gram negative bacilli and to analyse the antibiotic susceptibily pattern of the clinical isolates. It is necessary to know their incidence in the clinical isolates of the hospital, so as to formulate a policy of empirical therapy in high risk patients. (Coudron, 2005) Failure to identify them may lead to inappropriate therapy, treatment failure and may result in increased mortality.

MATERIALS AND METHODS

The present study of was carried out on a two hundred and fifty non-repetitive clinical isolates Gram negative bacilli from different clinical samples like urine, sputum, pus, blood and other samples collected from out-patients and in-patients admitted in the hospital. The isolates were identified by standard microbiological techniques. All the 250 Gram negative isolates were subjected to routine antibiotic susceptibility testing as per CLSI guidelines and were further tested for the production of carbapenemases.

Methods of Carbapenemase detection

Screening for carbapenemase (Clinical and laboratory standard institute, 2012**)**

All 250 isolates were screened for carbapenemase production using meropenem disc as per CLSI guidelines. (Clinical and laboratory standard institute, 2012)

Interpretation

Zone diameter of ≥ 23 mm and ≥16mm around the meropenem disc {HiMedia Laboratories,Mumbai, Maharashtra, India} for *Enterobacteriaceae* and *Pseudomonas aeruginosa* respectively was considered sensitive as per CLSI guidelines. (Clinical and laboratory standard institute, 2012)

Confirmatory tests for Carbapenemase production

Stock solution of Phenyl boronic acid (PBA) was prepared by dissolving 82.5 mg of PBA /ml of Dimethyl sulfoxide (Solvent for BA), so as to get the final concentration of 400µg/disc (Sigma Aldrich, India). A 0.5 M EDTA solution is prepared by dissolving 186.1 g of disodium EDTA. 2H2O in 1,000 ml of distilled water. The *p*H is adjusted to 8.0 by using NaOH (HI-MEDIA, Mumbai, India) and was sterilized by autoclaving. The meropenem resistant isolates were further tested for carbapenemase production by BA/EDTA disc potentiation test and Modified hodge test.

BA/EDTA disc potentiation test

Carbapenemase production was detected by a disc potentiation test using meropenem (MER) discs with Phenyl boronic acid (BA) and MER with Ethylene diamine tetra acetic acid (EDTA), and MER with both BA and EDTA. 5µl of a stock solution of phenyl boronic acid was dispensed onto MER discs. 10 µL of a stock solution of EDTA was dispensed onto MER discs. The discs were then dried and used within 60 min. The test was performed by inoculating Mueller–Hinton agar as given for the standard diffusion method and placing onto agar one disc of MER without any inhibitor and three discs of MER containing BA, EDTA and both BA+ EDTA as shown in Figure 1. The agar plates were incubated at 37ºC overnight. The diameter of the growth inhibitory zone around the meropenem disc with BA, EDTA, or BA+ EDTA was compared with that around the plain meropenem disc.

Interpretation

i. Production of KPC is considered when the zone around the MER disc with BA and the MER disc with both BA and EDTA is increased ≥ 5 mm compared with the zone around the disc containing MER alone. (Tsakris *et al*., 2010)
ii. Production of MBL is considered when the zone around MER disc with EDTA and the MER disc with both BA and EDTA is increased ≥ 5 mm compared with the zone around the disc containing MER alone. (Tsakris *et al*., 2010)
iii. Production of both KPC and MBL enzymes is considered when the zone around the MER disc with both BA and EDTA is increased ≥ 5 mm compared with the zone around the disc containing MER alone while the zones around the MER disc with BA and the MER disc with EDTA are increased ≥ 5 mm compared with the zone around the MER disc alone. Finally, when none of the three combined-disc tests is positive, the isolate is considered negative for MBL and KPC carbapenemase production. (Tsakris *et al*., 2010)

1) Modified Hodge test as per CLSI guidelines (Figure 2). (Clinical and laboratory standard institute, 2012)

RESULTS

We studied 250 clinical isolates of Gram negative bacilli from urine, sputum, pus, blood and other samples which included

various body fluids, CSF, endo-tracheal tube, vaginal swabs, conjunctival swab, ear discharge etc. Out of the 250 samples, majority of the isolates were from pus 86 (34.4%), followed by urine 72 (26.8%), sputum 40 (16%), blood 32 (12.8%) and other specimens 20 (8%). The most common organism isolated was *E coli* (44%) followed by *Klebsiella pneumoniae* (29.6%). The other Gram negative isolates include *Pseudomonas aeruginosa* (7.5%), *Klebsiella oxytoca* (6.4%), *Proteus mirabilis* (4.7%), *Citrobacter s*pecies (4.3%) and *Proteus vulgaris* (3.5%). As per CLSI criteria, of the 250 isolates, 55 (22%) were meropenem resistant, indicative of carbapenemase production. The remaining 195(78%) were sensitive to meropenem. KPC and MBL detected by BA/EDTA disc potentiation test is shown in Figure 3. Out of 55 meropenem resistant isolates, 46 (18.4%) were positive for carbapenemase production & 9 (3.6%) were negative for carbapenemase production by Modified Hodge test. As shown in table I, all the 8 (100%) KPC isolates were positive by MHT, whereas 24 (75%) of the MBL isolates were MHT positive. 8 (88.9%) co-producers of KPC and MBL was positive by MHT.6 meropenem resistant isolates which were neither KPC nor MBL showed positive results with MHT. The carbapenemases detected in various Gram negative bacilli is shown in table II.
Of the 250 isolates, 148 (59.2%) were resistant to ≥ 3 class of antibiotics tested in our study (Beta lactams, monobactams, flouroquinolones, aminoglycosides and carbapenems). [Multidrug resistant Gram negative bacilli (MDR GNB)[13]]. Out of these MDR GNB, 55 (22%) were meropenem resistant.

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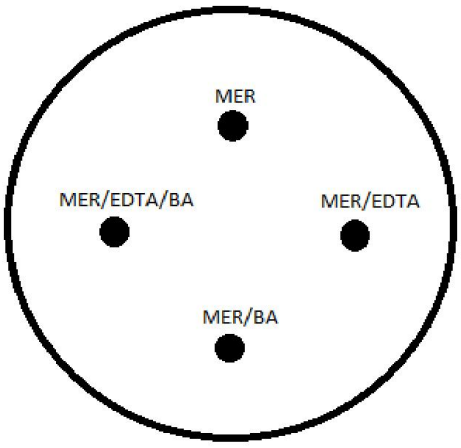

Figure 1. BA/ EDTA disc potentiation test

Out of 55 meropenem resistant isolates, 46 (18.4%) were positive for carbapenemase production & 9 (3.6%) were negative for carbapenemase production by Modified Hodge test. As shown in Table I, all the 8 (100%) KPC isolates were positive by MHT, whereas 24 (75%) of the MBL isolates were MHT positive. 8 (88.9%) co-producers of KPC and MBL was positive by MHT.6 meropenem resistant isolates which were neither KPC nor MBL showed positive results with MHT. The carbapenemases detected in various Gram negative bacilli is shown in Table II. Of the 250 isolates, 148 (59.2%) were resistant to ≥ 3 class of antibiotics tested in our study (Beta lactams, monobactams, flouroquinolones, aminoglycosides and carbapenems). [Multidrug resistant Gram negative bacilli (MDR GNB) (Magiorakos *et al*., 2012). Out of these MDR GNB, 55 (22%) were meropenem resistant.

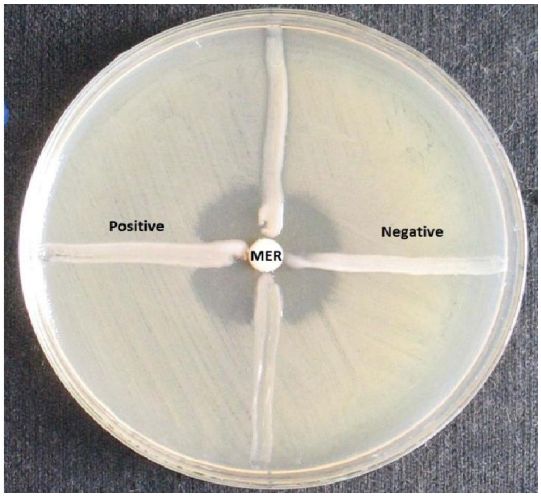

Figure 2. Modified Hodge test

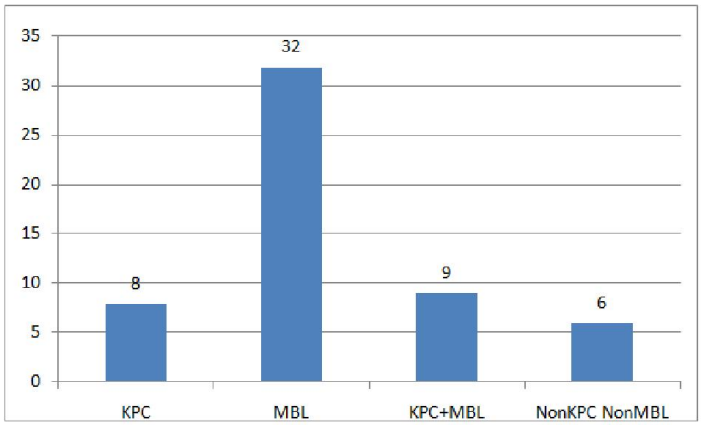

Figure 3. KPC and MBL detected by BA/EDTA disc potentiation test

Table I. KPC/MBL producers positive by CLSI recommended MHT

Carbapenemase	MHT		Total MER Resistant
	Positive (%)	Negative (%)	
KPC	8(100%)	0(0%)	8
MBL	24(75%)	8(25%)	32
KPC+ MBL	8(88.9%)	1(11.1%)	9
NonKPC NonMBL	6(100%)	0(0%)	6
Total (%)	46(83.6%)	9(16.4%)	55

The other Gram negative isolates include *Pseudomonas aeruginosa* (7.5%), *Klebsiella oxytoca* (6.4%), *Proteus mirabilis* (4.7%), *Citrobacter s*pecies (4.3%) and *Proteus vulgaris* (3.5%). As per CLSI criteria, of the 250 isolates, 55 (22%) were meropenem resistant, indicative of carbapenemase production.

Table II. Carbapenemases detected in various Gram negative bacilli

Organism	Carbapenemase (MER resistant)				MER sensitive	Total
	KPC (%)	MBL (%)	KPC+MBL (%)	nonKPC nonMBL (%)		
E.coli	2(25)	10(31.3)	1(11.1)	1(16.7)	96(49.2)	110
K.pneumoniae	3(37.5)	14(43.7)	4(44.5)	2(33.3)	51(26.1)	74
K.oxytoca	0(0)	4(12.5)	2(22.2)	0(0)	10(5.1)	16
P.mirabilis	0(0)	1(3.1)	1(11.1)	0(0)	10(5.1)	12
P.vulgaris	1(12.5)	0(0)	0(0)	1(16.7)	7(3.6)	9
Citrobacter spp.	1(12.5)	1(3.1)	1(11.1)	2(33.3)	5(2.6)	10
P.aeruginosa	1(12.5)	2(6.3)	0(0)	0(0)	16(8.3)	19
Total(%)	8(3.2%)	32(12.8%)	9(3.6%)	6(2.4%)	195(78%)	250

The remaining 195(78%) were sensitive to meropenem. KPC and MBL detected by BA/EDTA disc potentiation test is shown in Figure 3.

DISCUSSION

The incidence of nosocomial infections are showing a rising trend, which can be attributed to several practices followed in the hospitals. The β-lactam antibiotics are among the most frequently prescribed antibiotics world-wide, which are favoured because of their efficacy, broad spectrum and low toxicity. Indiscriminate use of these β-lactam antibiotics has lead to the selection of a variety of mutated forms of β-lactamases such as the ESBLs, AmpC-β-lactamases and carbapenemases. (Oberoi *et al*., 2013) These have emerged as the most worrisome resistance mechanism among the Gram negative bacteria, which poses a therapeutic challenge to the health care system. (Deshmukh *et al*., 2011) An alarming rise in the rates of the antibiotic resistance has now become a serious and an increasingly common public health concern, with severe implications. Screening for carbapenemase production was done as per CLSI guidelines. In our study, 55 (22%) meropenem resistant isolates were indicative of carbapenemase production. In various studies across the world varying resistance to imipenem and meropenem (4-60%) has been reported. (Gonlugur *et al*., 2004) Thus, practically, highly sensitive and specific methods for the detection of KPC-producers are needed.

In this regard, there is an increasing interest in the use of boronic acid compounds, which are β-lactamase inhibitors. (Tsakris *et al*., 2010) In our study, confirmatory tests were done using CLSI recommended MHT and BA/ EDTA disc potentiation test. 46 (18.4%) were positive for carbapenemase production by MHT and 49 (19.6%) were positive for carbapenemase production by BA/ EDTA test. All the 8 (100%) KPC producers and 8 (88.9%) co-producers of KPC and MBL were positive by MHT respectively. But 8 (25%) MBL producers and 1(11.1%) co-producer were negative by MHT (Table I). This is comparable to the study by Jesudason *et al.* in which EDTA disk synergy (EDS) could detect more carbapenemase and metallo-β- lactamase producers compared to MHT (Jesudason *et al*., 2005). EDS is a more sensitive method for detection of MBLs compared to MHT. (Noyal *et al*., 2009)

As per CLSI, it is suggested that the modified Hodge test should be used as a confirmatory test for carbapenemase production when the initial screening tests are indicative. (Clinical and laboratory standard institute, 2012) However, this test is often difficult to interpret, nonspecific and is only indicative of enzymatic activity of carbapenemase thus requiring PCR for differentiation. (Pournaras *et al*., 2010; Tsakris *et al*., 2010) Furthermore, there is increasing evidence that MHT gives false-positive results with CTX-M type ESBL positive or AmpC-hyperproducing *Enterobacteriaceae*. (Pournaras *et al*., 2010) Likewise in our present study 6 meropenem resistant isolates that were neither KPC nor MBL showed positive results with MHT but neither of these were co-producers of ESBL/AmpC. The limitation of our study was that these isolates needed further evaluation for other carbapenemases (other than KPC/MBL) by genotypic confirmation. However delayed reporting of potential carbapenemase producers to the clinicians should be avoided by performing phenotypic confirmation tests when genotypic confirmation are not available. (Gupta *et al*., 2012) In our study, we have confirmed KPC and MBL phenotypically by combining MHT along with carbapenemase inhibition tests to overcome false positive results with MHT alone and such a strategy can be followed to detect Class A carbapenemases phenotypically in *Enterobacteriaceae*. (Gupta *et al*., 2012) Overall, the meropenem-plus-inhibitor approach is more sensitive and specific than the other methods and despite its limitation of being unable to detect class D carbapenemases it has proven to be best compared to Modified Hodge Test. Regardless, molecular tests must be used to confirm the phenotypic results. (Christine *et al*., 2011)

Conclusion

The concomitant presence of several different types of β-lactamases in resistant *Enterobacteriaceae* makes it more difficult to detect individual mechanisms, as one mechanism can mask another. Phenotypic detection of these resistance mechanisms, though not confirmatory, is faster, far more cost effective, less labour intensive, and does not require a high level of technical expertise. Hence it is easier to perform and useful in exploring the treatment options of serious systemic infections by these pathogens. The BA/ EDTA disc potentiation test is better than MHT for detection of carbapenemase and MBL and is a satisfactory and inexpensive method for characterizing the type of carbapenemase when PCR is not readily available. Thus combined disc potentiation test could be used as a convenient screening method for carbapenemases. An early detection of β-lactamase producing organisms by simple screening methods can help in providing an appropriate antimicrobial therapy and in avoiding the development and dissemination of these multidrug resistant

strains. Antibiotic susceptibility pattern of the isolates showed that the majority were sensitive to carbapenems. The need of the hour is that every health care institution must develop its own antimicrobial stewardship program which is based on the local epidemiological data and international guidelines, to optimize the antimicrobial use among the hospitalized patients, to improve the patient outcomes, to ensure a cost-effective therapy and to reduce the adverse consequences of the antimicrobial use.

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